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Metabolic Engineering of Higher Plants to Produce Bio-industrial Oils

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Biography: David C. Taylor

David Taylor has been active for 22 years in the field of oilseed biochemistry, biotechnology, and oil modification at the National Research Council of Canada Plant Biotechnology Institute in Saskatoon, Saskatchewan. His research focuses on the creation of value-added oils as industrial feedstocks or for human consumption using biotechnology. David is also actively involved in research to improve the oil content of a number of oilseed crops including canola, *Brassica carinata* and flax. He is a strong advocate for adoption of *B. carinata* as a new platform crop for delivery of customized bio-industrial oils. He can be contacted by e-mail at david.taylor@nrc-cnrc.gc.ca

Biography: Mark A. Smith

Mark Smith is a Research Officer at the National Research Council Canada, Plant Biotechnology Institute in Saskatoon. Research conducted by his team focuses on trying to understand how plants make oil, what determines the fatty acid composition of oil and how this can be changed for specific end uses. He uses *Arabidopsis thaliana* and the yeast *Saccharomyces cerevisiae* as model systems, but also works with oilseed Brassicas, and with *Camelina sativa* (false flax). Target fatty acids include hydroxy fatty acids such as ricinoleic acid, saturated fatty acids and very long chain fatty acids. Mark obtained his B.Sc and Ph.D. from the University of Bristol in England. He has worked in Canada since 1997.

Biography: Pierre Fobert

Pierre Fobert is a Senior Research Officer at the NRC Plant Biotechnology Institute (PBI), where he has been for the past 16 years, and an Adjunct Professor of Biology at the University of Saskatchewan. Pierre received his Ph.D. in Plant Molecular Biology from Carleton University, Ottawa, Canada, in collaboration with the Eastern Cereal and Oilseed Research Centre of Agriculture and Agri-Food Canada. He joined the PBI after post-doctoral stages at the John Innes Centre and the Canadian Forestry Service. His group makes use of molecular biology, genomics and genetic approaches to study the transcriptional regulation underpinning traits of agronomic importance and manipulate gene expression for the purpose of crop improvement.

Biography: Elzbieta Mietkiewska

Elzbieta Mietkiewska conducts research on plant lipids in the Department of Agricultural, Food and Nutritional Sciences, University of Alberta, Canada. She received her Ph.D. in plant virology from the Plant Breeding and Acclimatization Institute in Poland. She has extensive experience in plant molecular biology, biochemistry and biotechnology. Over the last 12 years, she has been involved in project on genetic engineering of Brassica oilseed species to increase content of industrial and nutraceutic fatty acids. Her current research focuses on isolation and characterization of novel genes for increasing oil content in canola.

Biography: Randall J. Weselake

Randall is Professor and Tier I Canada Research Chair in Agricultural Lipid Biotechnology in the Department of Agricultural, Food and Nutritional Science at the University of Alberta (Edmonton, Alberta, Canada). He also serves as Scientific Director of the Alberta Ingenuity Phytola Centre which is developing plant oils for food and non-food applications. Randall was a Faculty member in the Department of Chemistry and Biochemistry at the University of Lethbridge (Alberta, Canada) from 1989-2004 where he served as Chair from 1996-1999. He has been involved in biochemical and molecular aspects of plant lipid research since 1987. Randall received his Ph.D. in plant biochemistry from the University of Manitoba (Winnipeg, Manitoba, Canada)

Abstract

Vegetable oils have enormous potential as alternatives and replacements for mineral oil in a myriad of industrial applications. Although our knowledge of the genes and biochemical pathways leading to the formation of plant oils allows for the potential to engineer a diverse array of lipid products in seed oils, this goal remains a challenge. This review first summarizes current industrial uses of plant oils while highlighting features that make them attractive for industrial applications and presents a general overview of seed oil biosynthesis. Thereafter, the review considers various metabolic engineering strategies to achieve desired fatty acid compositions of seed oils for industrial applications. Among the target modifications examined are fatty acid chain length, level of desaturation and the presence of novel functional groups, which are introduced within the endoplasmic reticulum. The role of triacylglycerol assembly in accommodating industrially useful fatty acyl groups is also addressed. In addition, a section is devoted to examining the manipulation of key steps in carbon flow to increase seed oil content. The concept of producing oil in vegetative tissues is also addressed, as is the development of ‘platforms’ crops for the production of metabolically engineered bio-industrial oils. Finally, this review examines the potential of waxes for industrial applications which require lubricants that are more stable to hydrolysis at higher temperatures.

Key Words

Bio-industrial oils, biodiesel, seed oil biosynthesis, membrane lipids, medium chain fatty acids, very long chain fatty acids, oil content, oleic acid, transcription factors, waxes, platform crops, *Brassica carinata*, *Camelina sativa*

Glossary

Carbon Credit - A carbon credit is a generic term for any tradable certificate or permit representing the right to emit one tonne of carbon dioxide or carbon dioxide equivalent.

Drying Oils – These oils are rich in polyunsaturated fatty acids and harden on exposure to air through oxidative polymerization.

Eukaryotic Pathway – See prokaryotic pathway.

Germplasm – Plant germplasm is living tissue from which new plants can be grown. Usually in the form of seed or growing plants, germplasm collections represent the genetic resources of an organism.

Identity Preservation – This represents a system of product separation for agricultural products involving all stages of production, handling and marketing. Identity preservation practises maintain integrity and purity of a product and also prevent the contamination of other products.

Prokaryotic Pathway – Plants use two distinct pathways for the synthesis of membrane glycerolipids. The prokaryotic pathway refers to the synthesis of lipids within the chloroplast (plastid), whereas the eukaryotic pathway begins with fatty acids exported from the chloroplast with glycerolipid assembly occurring predominantly in the endoplasmic reticulum.

List of Abbreviations

ABI3, ABSCISIC ACID INSENSITIVE3; ACCase, acetyl-CoA carboxylase; ACoASase, acyl-Coenzyme A synthetase; ER, endoplasmic reticulum; ACP, acyl-Coenzyme A-binding protein; ACP, acyl carrier protein; CoA, Coenzyme A; CPTase, cholinephosphotransferase; DAG, diacylglycerol; DES, fatty acyl-Coenzyme A desaturase; DGAT, diacylglycerol acyltransferase; FA, fatty acid; FAD, fatty acid desaturase; FAE, fatty acid elongation; FAS, fatty acid synthesis; FAT, fatty acid thioesterase; FUS3, FUSCA3; GPAT, *sn*-glycerol-3-phosphate acyltransferase; G3P, *sn*-glycerol-3-phosphate; HEAR, high erucic acid rapeseed; HOA, high oleic acid; *HS12*, *HIGH-LEVEL EXPRESSION OF SUCROSE INDUCIBLE GENE2*; KAS, β -ketoacyl-acyl carrier protein synthase; KCS, 3-ketoacyl-Coenzyme A synthase; LEC, LEAFY COTYLEDON; LPA, lysophosphatidic acid; LPAAT, lysophosphatidic acid acyltransferase; LPC, lysophosphatidylcholine; LPCAT, lysophosphatidylcholine acyltransferase; Mal-CoA, Malonyl-CoA; PA, phosphatidic acid; PC, phosphatidylcholine; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; *PKL*, *PICKLE*; PLA₂, phospholipase A₂; PUFA, polyunsaturated fatty acid; SAD, saturated acyl-Coenzyme A desaturase; SLC1-1, *Saccharomyces cerevisiae* lysophosphatidic acid acyltransferase; TAG, triacylglycerol; TF, transcription factor; VLCFA, very long chain fatty acid; WRI1, WRINKLE.

Note: Abbreviations for fatty acids are presented in the first time they are mentioned in the main body of the document and in figure 2.

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256.1 INTRODUCTION

Over the last 150 years, society has come to rely on fossil fuel-derived products for almost all aspects of life. Gas, oil and coal provide cheap energy, and are a source of raw materials for the manufacture of products ranging from lubricants and plastics to pharmaceuticals and cosmetics. World fossil fuel reserves are, however, a finite resource and the search for renewable replacements has been an ongoing activity for many years. Environmental concerns, and the introduction of financial incentives, such as “carbon credits” and “bio-fuel subsidies”, is now resulting in significant progress in the identification and development of renewable alternatives to petrochemicals. Plant oils, often referred to as vegetable oil, stand out as highly suitable replacements due to their chemical structure and because they have a long history of non-food use. Using these oils as global substitutes for mineral oil is, however, a challenging proposition, with the primary obstacles being price, availability and functionality. Modern plant biotechnology is a tool to address all of these factors.

The current review begins with a discussion of the characteristics of plants oils with a focus on those used for industrial applications. Thereafter, an overview of seed oil biosynthesis in plants is presented as background information pertinent to the next series of sections which focus on metabolic engineering of lipid biosynthesis to produce oils for specific applications. Oils for biodiesel production are discussed along with oils enriched in oleic acid (18:1 Δ^9 *cis*), very long chain monounsaturated fatty acids (FAs) and saturated FAs, for use in the soaps and detergent industries. A number of FA modifications can occur in the endoplasmic reticulum (ER) of plants producing unusual FAs with potential industrial applications. Thus, the review goes on to address the formation of FAs, such as hydroxy and epoxy FAs, and provides information on metabolic engineering strategies to incorporate these unusual FAs into the seed oil of major

oilseed crops. If plant oils are to be used for both food and non-food applications and meet the demands of an expanding world population with decreased dependence on petrochemicals, then it is also important to develop new strategies to increase global oil yield. An entire section is devoted to examining key steps in carbon flow and factors regulating carbon flow that have been manipulated to increase seed oil content. The concept of producing oil in vegetative and root tissue is also addressed as a possible means of developing new non-seed tissues for oil delivery. The review then moves on to discuss suitable crops which could serve as ‘platforms’ for the production of metabolically engineered industrial oils. Finally, wax biosynthesis and the potential for engineering crops to produce wax for various industrial applications are discussed.

256.2 PLANT STORAGE LIPIDS

Crude oil is a highly complex mixture of hydrocarbons, the major components being linear and branched alkanes, cycloalkanes, aromatics and alkenes. These are separated into various fractions during refining, and subjected to additional processing, such as thermal cracking and reforming, to produce fuels and chemical feed stocks. Plant oils are a much simpler form of reduced hydrocarbon, being predominantly composed of aliphatic carboxylic acids (FAs) esterified to glycerol to form of triacylglycerol (TAG; Figure 1A) with the FA components largely determining the properties of the oil. The major commercial oils only contain a small number of different FAs (Figure 2), but the distribution of these FAs within the TAG gives rise to complexity at a molecular level.

Exceptions to TAG as the major storage lipid molecule are found in a few species and these include liquid wax (wax esters) (Figure 1B) found in the seeds of Jojoba (*Simmondsia*

chinensis) and acylglycerol-estolides (Figure 1C), which are often a component of plant seed oils containing hydroxy FAs, such as members of the genus *Lesquerella* (Brassicaceae).

Compared to the volume of crude oil extracted every day, world vegetable oil production is modest. Data for the 2009/2010 cropping year estimated world production at close to 140 million metric tonnes (Tables 1 and 2). Of this, approximately 20% was used for non-food uses, including biofuel. By comparison, world production of crude oil in the first half of 2010 was estimated at close to 86 million barrels per day (1 barrel = 42 US gallons = 159 litres), with the majority being used for transportation fuel and energy generation. Only nine major plant species account for the majority of world seed oil production (Table 2); these include tropical species such as oil palm and coconut and annual temperate oilseeds such as rapeseed and sunflower. Within these species, many varieties are now being developed with oil FA compositions tailored for specific uses. The FA compositions of the oils of the most commonly grown commercial varieties are depicted in Table 2. Oil palm accounts for nearly one third of world oil production with the second largest production being from soybean, a crop grown primarily for protein, as its seed oil content is only around 20%. Plant oils have traditionally been considerably more expensive than crude oil. Recent increases in crude oil prices have narrowed this gap and sparked renewed interest in the use of vegetable oils for industrial uses.

256.3 INDUSTRIAL USES OF PLANT OILS

A list of important non-food uses of vegetable oil is given in Table 3. Recent publications on this topic include books such as *Industrial uses of Vegetable Oils* edited by Sevim Erhan (AOCS Press, ISBN 978-1-893997-84-4) and articles in the 2010 open access special issue of the *European Journal of Lipid Science and Technology*, volume 112, issue 1,

“Oil and fats as renewable resources for the chemical industry” [<http://online-library.wiley.com/doi/10.1002/ejlt.v112:1/issuetoc>]. Currently one of the major uses of plant oils is as a renewable fuel in the form of biodiesel (FA methyl esters) or Bio-SPK (see section 5). For applications such as lubricants and hydraulic fluids, plant oil can act as direct replacements for mineral oil, or require only minor chemical modification to replace a petrochemical-derived feedstock. Vegetable oil-based lubricants have the advantage of biodegradability, and superior lubricity, but generally require additives to increase oxidative stability, control viscosity at lower temperatures and to prevent hydrolytic cleavage of the ester bonds. Recent reviews have covered the development of vegetable oil as a biolubricant [1, 2].

As a chemical feedstock, the TAG molecule is usually cleaved to glycerol and FAs, or converted to alkyl-esters (usually methyl esters) and glycerol by transesterification. The utility of the FAs and esters is determined primarily by their chain length and functionality. For most common FAs, functionality is determined by the number and position of double bonds. These allow for the chemical modification of the acyl chain, for example by epoxidation, oxidative cleavage and metathesis to generate an array of oleochemicals [3, 4]. FAs with diverse functional groups exist in nature, although generally not in commercial oilseed crops, and many have potential value as new chemical feedstocks (Figure 2). Plant oils have received much attention as petrochemical replacements for the manufacture of polymers including polyurethanes, polyamides and epoxy resins. A wide range of processes have been developed utilizing chemically modified TAG or various FA or glycerol derived components [5, 6].

Although many new industrial uses for plant oils are being developed, some oil crops have traditionally been grown for non-food uses. Familiar examples are castor (*Ricinus*

communis), linseed (*Linum usitatissimum*), high erucic acid (22:1 Δ^{13} *cis*) rapeseed (HEAR) (*Brassica napus* varieties) and tung or “China oil” (*Aleurites fordii*, syn. *Vernicia fordii*). Many other minor oilseeds are cultivated throughout the world with some of the more important ones listed in Table 4. Plants grown for non-food uses generally produce FAs that differ from the common FAs in chain length or functionality. Castor bean, for example, is the only commercial source of ricinoleic acid (12-OH 18:1 Δ^9 *cis*), an unusual hydroxy FA (Figure 2) that makes up nearly 90% of seed FAs in this species. Although castor seeds are highly toxic, containing ricin and hyper-allergenic 2S albumins, castor oil commands a high price and is used in a wide variety of non-food applications from polymers to cosmetics [<http://onlinelibrary.wiley.com/doi/10.1002/ejlt.200900138/pdf>]. Currently, one of the largest users of castor oil is the chemical giant Arkema [<http://www.arkema.com/sites/group/en/home.page>] who manufacture Polyamide 11 or nylon 11, known commercially as Rilsan, from 11-aminoundecanoic acid, a product derived from the pyrolysis of ricinoleic acid. Rilsan is particularly suited to applications in natural gas pipelines and fuel tanks due to its resistance to hydrocarbons and lack of swelling when exposed to water. Castor oil also has medicinal and food uses, polyglycerol polyricinoleate for example is an ingredient in Twix bars (Mars Inc.).

Linseed oil and tung oil are both referred to as drying oils and are used primarily in the manufacture of surface coatings such as paints and varnishes as both oils readily oxidize and polymerize. Linseed oil is high (45-50%) in α -linolenic acid (18:3 $\Delta^9, 12, 15$ *cis*) whereas tung oil contains an unusual conjugated trienoic acid, α -eleostearic acid (18:3 Δ^9 *cis* $\Delta^{11}, 13$ *trans*) at levels of up to 80%. HEAR is grown commercially as the major source of erucic acid with *Crambe* (*Crambe abyssinica*) and *B. carinata* (see section 256.11) being developed as alternative

high erucic acid crops. Erucamide is the main chemical derivative of erucic acid and is widely used as a slip agent for plastic film. A second derivative, the 22 carbon saturated FA behenic acid (22:0) has multiple uses ranging from thermal film processing, where its sharp melting point is critical, to acting as a thickener in peanut butter. In addition, coconut and palm kernel oil have a long history as a source of the medium chain FA lauric acid (12:0) widely used in the manufacture of soaps and detergents.

Plant oils are already used in a wide variety of applications and have great potential as new renewable resources for the fuel and chemical sectors. Their widespread uptake by these industries is still limited by price and availability compared to petrochemical products. Physical properties are also an important factor in utilization. Plant oils are rarely uniform in FA composition, with individual TAG molecules containing a mixture of FAs. In addition, as indicated in Table 2, commercial oilseeds only produce a small number of different FAs. Research and commercialization activities aimed at optimizing seed oils for non-food uses have therefore focused largely on three areas; improving FA uniformity, tailoring oil composition towards specific requirements and increasing seed oil content.

256.4 SEED OIL BIOSYNTHESIS

C₂-C₁₈ FAs are synthesized in the plastid, whereas TAG is synthesized on the ER. Most of the discussion below is based on recent reviews of FA and TAG synthesis [7, 8; http://lipid-library.aocs.org/plantbio/fa_biosynth/index.htm]. An overview of FA and TAG synthesis in Brassica oilseed species is depicted in (Figure 3). Plastidial acetyl-CoA carboxylase (ACCase) is a multienzyme complex which catalyzes the formation of malonyl-CoA from acetyl-CoA and bicarbonate in an ATP-dependent process. The majority of acetyl-CoA utilized by ACCase is

produced through the catalytic action of plastidial pyruvate dehydrogenase. Malonyl-CoA, produced via the action of plastidial ACCase, provides two carbons for lengthening of the FA chain while attached to acyl carrier protein (ACP) of the fatty acid synthase complex. In plants, the FAS complex consists of several polypeptides which catalyze different reactions involved in FA synthesis. The successive addition of two carbon units from malonyl-CoA involves the sequential catalytic action of a condensing enzyme, a first reductase, then a dehydrase and finally a second reductase. The first condensation reaction is catalyzed by β -ketoacyl-acyl carrier protein synthase (KAS) III which requires acetyl-CoA and malonyl-CoA as substrates. Further elongation to a 16-carbon acyl chain from butyryl-ACP requires the catalytic action of KASI whereas production of stearyl (18:0)-ACP requires the action of KASII. Acyl-ACPs can be used directly as substrates for the synthesis of plastidial lipids in the “prokaryotic pathway”. Alternatively, acyl-ACPs can be hydrolyzed through the action of fatty acid thioesterases (FATs; acyl hydrolases) and exported across the plastidial envelope where they are reactivated to acyl-Coenzyme A (CoA) thioesters via the action of acyl-CoA synthetase to serve as substrates in the “eukaryotic pathway” of lipid synthesis. Monounsaturated FAs are produced in the plastid through the action of a soluble acyl-ACP desaturase. In Arabidopsis and many major oilseed crops, a stearyl-ACP desaturase catalyzes the conversion of the stearyl moiety into the oleoyl moiety.

Further elongation of FAs can occur on the ER through the action of membrane-bound elongases (described in section 256.7) which require acyl-CoA and malonyl-CoA as co-substrates. Cytosolic malonyl-CoA used in FA elongation is produced through the action of a cytosolic ACCase consisting of a large single polypeptide (multifunctional protein). In HEAR, erucoyl-CoA is produced from oleoyl-CoA through two successive additions of two carbon

fragments provided by malonyl-CoA. The elimination of erucic acid from modern day Brassica oilseed species (canola) was due to the selection of germplasm with a gene encoding an inactivated elongase [<http://onlinelibrary.wiley.com/doi/10.1046/j.1432-1033.2002.03270.x/pdf>].

TAG assembly in the ER is intimately associated with membrane metabolism and involves the action of several membrane-bound enzymes [8] (Figure 3). It is generally accepted that TAG assembly leads to the formation of lipid bodies ranging from 0.2 to 2 microns in diameter which “pinch off” of the ER and end up in the cytosol as “oil bodies”. The TAG is left surrounded by a “half-unit membrane” which contains a number of embedded lipid body proteins. *sn*-Glycerol-3-phosphate (G3P) serves as the glycerol backbone for TAG assembly. G3P is produced from dihydroxyacetone phosphate through the action of *sn*-glycerol-3-phosphate dehydrogenase. *sn*-Glycerol-3-phosphate acyltransferase (GPAT) catalyzes the acyl-CoA-dependent acylation of G3P to produce lysophosphatidic acid (LPA). A second acyl-CoA-dependent acylation to produce phosphatidic acid (PA) is catalyzed by lysophosphatidic acid acyltransferase (LPAAT). PA phosphatase catalyzes the hydrolysis of PA to produce *sn*-1,2-diacylglycerol (DAG) which is the substrate for the final acyl-CoA-dependent acylation of the glycerol backbone catalyzed by diacylglycerol acyltransferase (DGAT). The substrate selectivity properties of the enzymes in the mainstream of TAG assembly can influence the FA composition of TAG. For example, a DGAT with increased preference for ricinoleoyl-CoA can contribute to the enrichment of ricinoleic acid in the *sn*-3 position of TAG [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2908398/pdf/nihms217608.pdf>].

Other reactions affecting TAG accumulation involve membrane metabolism and acyl editing. DAG produced from acylation of the glycerol backbone can also be converted to

phosphatidylcholine (PC) through the catalytic action of cholinephosphotransferase (CPTase). In turn, PC can serve as a substrate for the synthesis of polyunsaturated fatty acids (PUFAs) catalyzed by membrane-bound fatty acid desaturases (ie., FAD2 & FAD3). Some modified FAs, such as ricinoleic acid, are also produced on PC [9] with a hydroxylase enzyme which is essentially an evolutionarily-modified FAD2. In a recent study with *Arabidopsis*, a phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) was shown to catalyze the transfer of the phosphocholine headgroup from PC to DAG [10]. This reaction provides a reversible route for transferring oleoyl moieties into PC and removing PUFA acyl chains from PC for use in TAG assembly. These investigators further indicated that PDCT could potentially represent an important enzyme target for enriching industrially important FAs in TAG. In addition, suppression of *PDCT* expression might lead to seed oil that is more oxidatively stable for biodiesel applications. In recent study with developing soybean embryos, kinetically distinct DAG pools were identified during oil accumulation [11]. DAG used in TAG assembly was derived from PC whereas DAG produced through *de novo* synthesis (Kennedy pathway) was mainly used for PC production.

Phospholipid:diacylglycerol acyltransferase (PDAT), lysophosphatidylcholine acyltransferase (LPCAT) and phospholipase A₂ (PLA₂) catalyze other reactions that can potentially influence the movement of PUFA or modified FA into TAG. PDAT catalyzes the production of TAG through a acyl-CoA-independent process which uses PC as an acyl donor [12, 13]. Thus, PUFAs or other modified FA chains, produced on PC by the catalytic action of enzymes such as desaturases, hydroxylases and epoxidases, could potentially be transferred to TAG via the action of PDAT. A recent study using *DGAT1* and *PDAT1* mutants in *Arabidopsis* indicates that these two enzymes have overlapping functions in TAG assembly in both seeds and

in pollen [13]. PDAT from yeast can also catalyze the transfer of acyl groups between two molecules of DAG to generate TAG and monoacylglycerol [<http://www.ncbi.nlm.nih.gov/pubmed/18037386>]. LPCAT catalyzes acyl-exchange at the *sn*-2 position of PC with the acyl-CoA pool and thus leads to an enrichment of PUFA-CoAs in the the acyl-CoA pool. Thus, there are new opportunities for incorporation of PUFA or other modified FAs into TAG. PLA₂, which catalyzes the hydrolysis of acyl groups at the *sn*-2 position of PC, may produce liberated PUFAs or modified FAs for activation by the action of acyl-CoA synthetase. The lysophosphatidylcholine (LPC) left behind after deacylation of PC catalyzed by PDAT or PLA₂ could then be reconverted to PC through the forward reaction catalyzed by LPCAT.

Although PUFAs are generally produced at the level of PC, there are species which are capable of producing PUFAs using monounsaturated acyl-CoAs as substrates. For example, *Limnanthes* sp. (e.g., meadowfoam) contains a Δ 5 fatty acyl-Coenzyme A desaturase (DES 5) which can catalyze the conversion of erucoyl-CoA into Δ 5, Δ 13 docosadienoyl-(22:1 Δ 5, 13 *cis*)-CoA (see section 256.8; <http://www.ncbi.nlm.nih.gov/pubmed/7294800>; Figure 3). This enzyme can also catalyze the formation of Δ 5 eicosenoyl (20:1 Δ 5 *cis*)-CoA from the saturated fatty acyl-CoA, eicosanoyl (20:0)-CoA, which is produced by elongation of palmitoyl (16:0)-CoA (<http://www.ncbi.nlm.nih.gov/pmc/articles-/PMC59139/pdf/pp000243.pdf>). Δ 5 Eicosenoyl- and Δ 5, Δ 13 docosadienoyl-CoA would then contribute to the acyl-CoA pool and serve as substrates for acyl-CoA-dependent acyltransferases.

Acyl-Coenzyme A-binding proteins (ACBPs) might modulate storage lipid synthesis by regulating the availability of acyl-CoAs [11, 14]. Expression of a cDNA encoding cytosolic 10 kDa ACBP during seed development in *Arabidopsis* has been shown to result in oil with

enhanced PUFA content at the expense of FA elongation [15]. When these observations were combined with *in vitro* studies on the effect of this *ACBP* transgene expression on LPCAT action, it was concluded that ACBP can affect the equilibrium between metabolically-active acyl pools involved in FA modifications and TAG assembly.

256.5 OILS FOR BIODIESEL PRODUCTION

Due to increasing energy demands coupled with environmental awareness related to climate change, particularly as caused by the use of fossil fuels, it has become necessary to develop alternative fuels as well as renewable sources of energy [16, 17]. Biodiesel has been reported to be a good substitute for conventional petrol-based diesel [<http://online-library.wiley.com/doi/10.1002/ejlt.200900078/abstract>]. Most developed countries are moving from voluntary to obligatory legislation to increase the market share of biodiesel within the transport sector. For instance, the European Commission for the European transport sector set up a mandatory biofuel target of 10% of the fuel market by 2020 [16].

According to the US Standard Specification for Biodiesel (ASTM 6751-02), biodiesel is defined as a fuel comprised of mono-alkyl esters of long-chain FAs derived from vegetable oils or animal fats. Currently, the most established pathway for biodiesel production is via the alkali-catalyzed transesterification of plant oil or animal fat with methanol, in the presence of acid or alkali to produce FA methyl esters and glycerol [18]. Transesterification is necessary to reduce the typically higher viscosity of plant TAGs to a level comparable with conventional diesel [19]. Biodiesel offers a number of advantages over standard fossil fuels such as enhanced biodegradation, increased flashpoint, reduced toxicity, increased lubricity and lower emissions

[19]. The biggest advantage to using biodiesel at the present time is that it is a sustainable source of liquid fuels and is “neutral” with respect to the production of carbon dioxide.

Despite these advantages of biodiesel, there are also some significant drawbacks that have limited its commercial application [20]. In addition to the cost, limited adaptation is attributed to the oxidative instability of biodiesel derived from vegetable oils, a significant high viscosity of unblended biodiesel and reduced cold flow properties when compared with petroleum diesel. There is also an inverse correlation between cold flow and oxidative stability that affects biodiesel characteristics. Properties of biodiesel are dependent on the FA composition of the plant oil used for its production and these properties can be improved by altering FA composition of the oil [19]. Improving cold-temperature flow characteristics requires a fuel with low saturated FA levels whereas increasing oxidative stability and reducing nitrous oxide emissions requires decreasing the proportions PUFAs.

There are a number of potential feed stocks available for use in biodiesel production. These include vegetable oils such as soybean, corn, sunflower, cottonseed, peanut, canola and rapeseed. Considering the FA requirements, a plant oil high in monounsaturated FAs, such as oleate or palmitoleate (16:1 Δ 9 *cis*), and low in both saturated FAs and PUFAs is a good target for the production of high quality biodiesel. In Europe, where the majority of biodiesel is produced from rapeseed oil, new varieties with oleic acid content over 60% were developed as a result of experimental mutagenesis combined with conventional selection (see Section 6). For instance, oil of varieties such as “Splendor” or “Nexera” are characterized by oleic acid contents of more than 75% and linoleic acid (18:2 Δ 9, 12 *cis*) contents of less than 3%, making them well-suited for biodiesel production [21]. Studies have also confirmed that HEAR oil and its

derivatives, high in very long chain FAs, have a higher energy potential than low erucic oil [<http://www.springerlink.com/content/00915968j0270m77/>]. HEAR oils, including those from *B. carinata*, are more suitable for biodiesel production when compared to low erucic Brassica oils because the iodine value is lower and within the European Union specifications [<http://www.springerlink.com/content/c57wnl36ll4v5q05/>]. As documented below, some genetically-modified *B. carinata* lines have about 75% mono-unsaturated FAs produced by over-expressing elongase genes and silencing desaturase genes.

In the US, 3.6% of soybean oil production is devoted to industrial applications (approximately 300 million kg), of which 1.5% (4.5 million kg) is used for biodiesel [<http://www.unitedsoybean.org>]. Using a transgenic approach, soybean lines with high levels of oleic acid and low levels of saturated FAs and PUFAs were developed by down regulation of two genes: *FAD2-1* encoding a Δ -12 desaturase and *FatB* encoding palmitoyl-ACP thioesterase [<http://onlinelibrary.wiley.com/doi/10.1046/j.1365-313X.2002.01283.x/pdf>]. This transformation resulted in development of soybean lines with oleic acid levels of 85% and saturated FA levels below 6% compared to the wild-type levels of 18% and 13 %, respectively.

Over the last few years, production of biodiesel has expanded in both the European Union and the USA. In 2006, European countries produced approximately 5.6 billion liters of biodiesel compared to 0.9 billion liters produced in the USA [19]. The high cost of biodiesel production is significantly affected by the cost of the input oil. Recently, observed increases in the proportion of plant oils used for the production of biodiesel have already contributed to higher prices of vegetable oil, not only making biodiesel production more expensive, but also having an impact on food prices [19]. Further channelling of the supply of edible oils to biodiesel

production could be problematic as there is a potential conflict between producing crops for use as energy as opposed to food. Therefore, there is a need to develop alternative sources that produce non-edible oils that can be cultivated on marginal lands or require only a minimal amount of land for its oil production [22].

Recent studies have explored biodiesel production from less common oil sources including tobacco, pongamia, jatropha and rubber seeds [23]. Acetyl-rich TAGs are an interesting target: these are modified TAGs with a short two-carbon acetyl group rather than a longer acyl chain at the *sn*-3 position, resulting in an oil of lower viscosity that could potentially replace No. 4 diesel (a heavier grade). Acetyl-rich TAGs could potentially be used directly in certain applications without the need to generate FA methyl esters. These unusual TAGs are found in the seeds of members of the genus *Euonymus* where they can form up to 98% of the seed FAs. In an effort to engineer crops containing *sn*-3 acetyl groups, a divergent *DGAT* gene was cloned from *Euonymus alatus*, which is also known as “burning bush”. [\[http://www.pnas.org/content/early/2010/04/26/1001707107.full.pdf\]](http://www.pnas.org/content/early/2010/04/26/1001707107.full.pdf). Expression of the cDNA encoding this DGAT under the control of a strong, seed-specific promoter in *Arabidopsis* resulted in the accumulation of up to 40% acetyl-TAGs in the total seed TAG fraction from the transgenic lines. This finding is promising for introducing this gene into other oilseed crops to allow the evaluation of these unusual TAGs for biofuel and other applications.

Another attractive approach to increase overall yield of oils for biodiesel production is to engineer oil accumulation in vegetative tissues, such as leaves (discussed in Section 10.3). In addition, used rapeseed oil and other waste oils could be an alternative solution for biodiesel

production after application of the proper procedures for oil purification [24; http://teenet.tei.or.th/Knowledge/Paper/inform_magazine.pdf].

A more recent product to enter the bio-fuel market is Bio-Derived Synthetic Paraffinic Kerosene, referred to as Bio-SPK or Bio-jet [<http://www.safug.org/docs/biofuel-testing-summary.pdf>]. This and similar products are synthesized by the hydrogenation and cracking of vegetable oils. In comparison to Biodiesel, Bio-SPK has the advantages that it is feedstock-independent and uses the entire TAG molecule, so glycerol is not a by-product. However, the process is more energy intensive and requires a higher investment in infrastructure. By reaction with hydrogen, TAG molecules are converted to alkanes, CO₂ and water. A second reaction isomerizes and cracks the alkanes to produce smaller and often highly branched molecules that are similar in composition to petroleum derived aviation fuel. Although any plant oil can be utilized as a feedstock, the engineering of high oleate oils is beneficial to this industry as less H₂ is required for the initial hydrogenation step.

256.6 HIGH-OLEIC ACID OILS

A major objective in recent oilseed breeding projects has been the development of high oleic acid (HOA) lines in which the oleic acid content exceeds 70% of total seed FAs. Although originally aimed at the food market, HOA oils are of value as an industrial feedstock. Traditional breeding approaches have involved the identification of plants with altered seed oil FA profiles, often generated by mutagenesis, and combinations of these lines were eventually used to produce the desired HOA phenotype. This approach has been successfully applied to crops such as safflower (*Carthamus tinctorius*) and sunflower (*Helianthus annuus*), but has not been universally successful in oilseeds. In soybean, for example, mid-level oleic acid lines have been

identified, but the HOA phenotype is associated with reduced yield (yield drag), is strongly influenced by growth temperature and requires pyramiding of multiple alleles.

As shown in Figure 3, oleic acid is the predominant FA exported from the plastid during seed development in most oilseed crops, with further desaturation and/or elongation giving rise to the PUFAs and very long chain fatty acids (VLCFAs), respectively. Silencing of enzymes of oleate modification (as described in Section 4) specifically in the developing seed, rather than in the entire plant, has been shown to be a highly effective strategy to generate a HOA profile, and demonstrates the advantages and precision of genetic engineering over more traditional approaches. Seed-specific post transcriptional gene silencing is part of the mechanism that has been used to generate HOA soybeans that do not suffer from yield drag and which will soon be in commercial production [<http://www.plenish.com/>]. A further advantage of this approach is that the transgene is inherited as a single dominant trait, which will greatly simplify its incorporation into elite breeding lines [<http://www.plantphysiol.org/cgi/reprint/151/3/1030>].

As described later in this review, engineering the HOA oilseed profile can be compared to “picking the low hanging fruit” when compared to the engineering of oils enriched in other strategic industrial FAs. In most other cases, the simple silencing of endogenous genes will not produce the desired phenotype.

256.7 ENGINEERING OILS ENRICHED IN VERY LONG CHAIN FATTY ACIDS

VLCFAs are those that contain more than 18 carbon atoms and are common components of plant waxes and seed oils in a number of plant families including the Cruciferaeae, Limnantheceae, Simondsia and Tropaeolaceae [[http://www.ncbi.nlm.nih.gov/pmc-articles/PMC161098/pdf/080281.pdf](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC161098/pdf/080281.pdf); <http://www.plant-physiol.org/cgi/reprint/136/1/2665>;

<http://pubs.acs.org/doi/abs/10.1021/jf047939e>]. Erucic acid is the major VLCFA in the seed oil from HEAR *B. napus* cultivars, accounting for 45-55% of the total FAs [<http://www.springer-link.com/content/m021647r02w6x203/>]. Nervonic acid (24:1 Δ^{15} *cis*) is another strategic VLCFA and is found in the seed oils of only a few plants including *Lunaria* spp. (money plant), borage, hemp, *Acer truncatum* (purpleblow maple), *Tropaeolum speciosum* (flame flower) and *Cardamine graeca* (bittercress).

A strategic goal of our research is to modify HEAR germplasm of the Brassicaceae to increase the content of erucic and nervonic acids in the seed oil for industrial niche markets [25, 26]. HEAR cultivars are of high interest for industrial purposes because erucic acid is a valuable feedstock with more than 1000 potential or patented industrial applications [27]. Currently, the major derivative of erucic acid is erucamide, which is used as a surface-active additive in coatings and in the production of plastic films as an anti-block or slip-promoting agent. Many other applications are foreseen for erucic acid and its hydrogenated derivative behenic acid including production of lubricants, detergents, film processing agents and coatings along with cosmetics and pharmaceuticals [28]. Nervonic acid has applications similar to erucic acid, as an industrial feedstock [26] (see Table 5).

For many of these industrial uses, the economics are limited by the proportion of erucic or nervonic acid in the seed oil. With respect to the market for HEAR oil, it is estimated that about 80,000 tons is used annually worldwide for lubricants, plastics, lacquers and detergents [<http://www.gov.mb.ca/agriculture/research/ardi/projects/98-022.html>] Additionally, the European market for HEAR oil in 2005 was estimated at 55,000 MT with an annual growth rate of 4 to 5 percent [<http://www.ienica.net/crops/crambe.pdf>]. A Brassica cultivar containing erucic

acid or nervonic acid at levels approaching 80% would substantially reduce the cost of producing these VLCFAs and their derivatives and could meet the forecast increase in demand for these seed oil products as renewable, environmentally friendly industrial feedstocks [25, 28;

<http://www.springerlink.com/content/xypx3mk4m138l7cj/>]. In addition, the engineering of HEAR Brassicaceae to produce seed oils containing substantial trierucin would lend the oil to a wide range of new applications, especially as a lubricant which is very stable at high temperatures [29].

VLCFAs are synthesized by a microsomal fatty acid elongation (FAE) complex using acyl-CoA substrates from a cytoplasmic pool maintained by de novo lipid biosynthesis in plastids [7; see Figure 3]. Each cycle of FA elongation adds two carbon units to the acyl chain and involves four reactions: firstly, malonyl-CoA and long chain acyl-CoA are condensed by a 3-ketoacyl-Coenzyme A synthase (KCS, often designated FAE): the resulting 3-ketoacyl-CoA is then reduced by the action of a 3-ketoacyl-CoA reductase resulting in the synthesis of a 3-hydroxyacyl-CoA. Subsequently, 3-hydroxyacyl-CoA is dehydrated to 2-enoyl-CoA which is then reduced by second reductase to form the elongated acyl-CoA. Oleoyl-CoA is the substrate for production of eicosenoyl-CoA (20:1 Δ^{11} *cis*), which is then successively elongated to erucoyl-CoA and then nervonoyl-CoA by cycling through the FAE complex.

Over the past decade, progress in understanding VLCFA biosynthesis has been achieved by cloning *KCS* genes from different plants and performing functional expression studies [e.g., <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC161098/pdf/080281.pdf>]. These types of studies have provided evidence that KCS activity can limit the flow of carbon in VLCFA production [<http://www.plantphysiol.org/cgi/reprint/136/1/2665>] and its acyl chain-length specificity

determines the ultimate elongated acyl-CoA product. Due to the membrane-bound nature of the KCS (FAE) protein our knowledge of the properties and regulation of this enzyme are still limited [<http://onlinelibrary.wiley.com/doi/10.1002/ejlt.200590024/abstract>].

In searching for new sources of strategic *KCS* genes to engineer high VLCFA oils, *Crambe abyssinica* and *Cardamine graeca* were selected to enhance the proportions of erucic and nervonic acids, respectively: The seed oil of *C. abyssinica* is distinct from other Cruciferae because of its very high proportion of erucic acid, up to 60%, while *Cardamine graeca* has 45% nervonic acid, the highest known to date [26].

Over-expression of the *C. abyssinica KCS* gene in *B. carinata* resulted in a substantial increase in the proportion of erucic acid in seeds compared to the wild type control [<http://onlinelibrary.wiley.com/doi/10.1111/j.1467-7652.2007.00268.x/abstract>]. The seed oil from these transgenic lines exhibited erucic acid proportions as high as 52%, a net relative increase of 40% compared to wild type. Equally effective was over-expression of the *Cardamine graeca KCS* gene in *B. carinata*, which resulted in an increase of nervonic acid from 1.5% in the wild type to 45% in the best transgenic lines.

The synthesis of erucic and nervonic acids in transgenic *B. carinata* plants was probably, in part, limited by the smaller microsomal pool of oleoyl- moieties (7-8%) available for elongation. As pointed out previously by Bao et al. [[http://www.plantphysiol.org/cgi/reprint-118/1/183](http://www.plantphysiol.org/cgi/reprint/118/1/183)], and subsequently by Jadhav et al. [25], the fact that oleoyl-CoA is a critical intermediate for both membrane and storage lipid, FAs might be a factor that limits the availability of oleoyl moiety for elongation. The oleate desaturase, FAD2, is one of the crucial enzymes for the production of PUFAs in plants [7]. As we have shown, by altering the level of

FAD2 gene expression using antisense and co-suppression approaches, it was possible to increase the pool of oleoyl moieties available for elongation to moderately enhance production of erucic acid in *B. carinata* seeds [25]. The antisense and co-suppression strategies, however, have variable and unpredictable effectiveness and require the production of large populations of transgenic plants to obtain a reasonable number of lines showing sufficient levels of target gene suppression [<http://www.plantphysiol.org/cgi/reprint/129/4/1732>].

The discovery that RNA interference in plants is mediated by sequence-specific degradation of dsRNA has led to the development of highly efficient methods of post-transcriptional gene silencing. Constructs specially designed to express dsRNA in plants in the form of self-complementary hairpin RNA (hpRNA) elicit a high degree and frequency of post-transcriptional gene silencing of endogenous genes [<http://www.pi.csiro.au/RNAi/>; <http://www.-plantphysiol.org/cgi/rapidpdf/pp.006353v1.pdf>; <http://onlinelibrary.wiley.com/doi/10.1046/j.-1365-313X.2001.01105.x/pdf>]. In our studies, we used a partial 3'-UTR of the seed-specific *B. carinata* *FAD2* gene to prepare an intron-spliced hpRNA construct to silence the seed *FAD2* gene and subsequently, to increase the pool of oleoyl moieties available for elongation. This strategy, when combined with over-expression of *C. abyssinica* *KCS* resulted in a further increase in the content of erucic acid in *B. carinata* to as high as 58%, a 45% proportional increase over wild type levels of 40% [30, 31]. The overall proportion of mono-unsaturated FAs in this oil was >70%. We anticipate a similar result using this strategy with *B. carinata* transgenics producing nervonic acid.

Another bottleneck that needs to be addressed to further increase VLCFA content in *B. carinata* is the limitation caused by very low ability to incorporate erucoyl- or nervonoyl

moieties into *sn*-2 position of TAG by the endogenous Brassica LPAAT. As in other HEAR, with *B. carinata*, less than 2% of erucic acid (and negligible nervonic acid) is found at the *sn*-2 position on the glycerol backbone [27]. This restricts the level of erucic acid or other VLCFAs in the HEAR seed to a theoretical limit of 66% [<http://www.informaworld.com/smpp/content~db=all~content=a905098171~frm=abslink>]. Increasing the erucic acid level of HEAR *B. napus* has been attempted by over expressing *LPAAT* genes from *Limnanthes* sp. [[http://onlinelibrary.wiley.com/doi/10.1002/\(SICI\)1521-4133\(19985\)100:4/5%3C161::AID-LIPI161%3E3.0.CO;2-P/abstract](http://onlinelibrary.wiley.com/doi/10.1002/(SICI)1521-4133(19985)100:4/5%3C161::AID-LIPI161%3E3.0.CO;2-P/abstract)]. This approach resulted in an increased proportion of erucoyl moieties at the *sn*-2 position up to 41%. Modification of the *sn*-2 FA composition, however, did not enhance the total erucic acid content [<http://www.springerlink.com/content/jk702265696-1u1h8/>; <http://www.plantphysiol.org/cgi/-reprint/109/4/1389>]. These data indicated that in HEAR expressing *Limnanthes LPAATs*, the total erucic acid content was most likely limited by the activities of microsomal KCS involved in the synthesis of erucoyl-CoA. More successful was a study with a yeast mutant *SLC1-1* (*LPAAT*) gene described by Zou et al. [<http://www.plantcell.org/cgi/reprint/9/6/909.pdf>]. In contrast to *Limnanthes LPAATs*, expression of the yeast *SLC1-1* affected not only the stereochemical composition of seed oil, but also total erucic acid and oil content. The total content of erucic acid increased up to 56% compared to 45% in the control.

Considering our previous work on over expression of yeast *SLC1-1* in *B. napus*, we have transformed *B. carinata* with a construct carrying the *SCL1-1* gene + Crambe KCS under control of the napin promoter [26]. This recent work has shown that co-expression of these two genes results in an increase in both oil and erucic acid content. For instance, erucic acid content increased from 41% in the control line up to 47% in the best transgenic line. Also, significant

increases in overall oil content were observed, up to a 25% relative increase above the control level.

In view of these results, it is clear that by altering expression of one or two genes it is impossible to increase erucic or nervonic acid levels over 66% in *B. carinata* seed oil. A recent study by Nath et al. [32] has shown that combining alleles of *B. napus* related to low polyunsaturated oils (i.e., HOA) with the transgenic co-expression of the *L. douglasii LPAAT2* and the *B. napus FAE1* (encoding KCS) led to the development of a *B. napus* line with a seed oil erucic acid content of 72%. Thus, additional genes will have to be introduced into *B. carinata* to address all the bottlenecks to increase erucic or nervonic acid content over 70%. A recently isolated and characterized *LPAAT2* gene from *Tropaeolum majus* (nasturtium) is a good candidate to engineer further increase VLCFAs in transgenic *B. carinata* lines developed in our group [<http://bentham.-org/open/topsj/openaccess2.htm>]. Currently, experiments are underway to re-transform the *B. carinata* line carrying the Crambe *KCS* + hpRNA *FAD2* dual transgene as well as the *B. carinata* line carrying the *Cardamine KCS* transgene, with the nasturtium *LPAAT2*.

Another VLCFA of potential industrial interest is docosadienoic acid (22:2 Δ^5 , 13 *cis*). This unique diene is found in the oils of Limnathaceae (e.g., meadowfoam) and is superior compared to other PUFAs with respect to resistance to oxidation due to its widely-spaced, non-methylene- interrupted double bonds. There are niche market applications that have been identified for use of docosadienoic acid as a substrate for generating estolides, which can be used to synthesize hydroxy FA feedstocks, and to produce dimer acids, esters and amides for use as lubricants [<http://etmd.nal.usda.gov/bitstream/10113/24375/1/IND91036005.pdf>; <http://ddr-.nal.usda.gov/bitstream/10113/25412/1/IND93044955.pdf>]. The FA is synthesized in *Limnanthes*

via the catalytic action of DES 5 (see Figure 3). The *Des5* gene from *Limnanthes* has been cloned and expressed in *B. carinata* resulting in yields of docosadienoic acid which were about the same as that found in native meadowfoam oil [25]. However, if this *Des5* gene can be introduced into the prototype high erucic *B. carinata* lines with silenced *FAD2* + Crambe *KCS*, the transgenic oil will probably exhibit substantially greater proportions of docosadienoic acid.

We are advocating that *B. carinata* be developed as an alternative crop platform for industrial oil production and high-erucic oils, in particular [26]. *B. carinata* is easily transformed at very high efficiency, is highly disease (e.g., blackleg)-resistant and is drought-resistant, being amenable to growth in hotter and drier regions, such as the brown soil areas of southern Saskatchewan. While *B. carinata* has an outcrossing rate of 20-30% within its own species, a rate typical within each species of the Brassicaceae, it fortunately also has a very low frequency of out-crossing to canola [26], and therefore poses a lower risk of contaminating oils destined for the food chain. New breeding lines of *B. carinata* with higher oil and low glucosinolate content are currently being developed at Agriculture and Agri-Food Canada (K. Falk, personal communication) and will provide excellent germplasm for production of high erucic and other industrial oils.

256.8 OILS FOR THE DETERGENT INDUSTRY

Palm and coconut oils serve as a major source of medium-chain FA feedstock for the detergent industry. Researchers at Calgene have engineered canola (*B. napus*) to form tropical-type oil' enriched in medium chain FAs [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC59311/pdf/739.pdf>; <http://www.patentstorm.us/patents/5750481/description.html>]. Oil with > 50% lauric acid (12:0) was obtained by co-expressing a cDNA encoding lauroyl-ACP

thioesterase from the California bay laurel (*Umbellularia californica*) with a cDNA encoding coconut (*Cocos nucifera*) LPAAT with enhanced preference for lauroyl-CoA. The California bay laurel thioester catalyzed the preferential release of lauric acid from the FA synthase complex in the plastid to the ER compartment; converted to lauroyl-CoA, it was utilized by the coconut LPAAT to esterify lauroyl moieties at the *sn*-2 position of the glycerol backbone. The bay thioesterase had to compete with an endogenous thioesterase which released 18-carbon FAs from the FA synthase complex, whereas the coconut LPAAT competed with endogenous LPAAT activity which discriminated against saturated FAs.

256.9 INCREASING FUNCTIONALITY: FATTY ACID MODIFICATION IN MEMBRANE LIPIDS

Many of the FAs of interest as industrial feedstocks are synthesized in plants by the modification of FAs esterified to the membrane phospholipid PC (see Figure 3). These FAs are then removed from the intracellular membranes and incorporated into storage TAG by processes that are not fully understood. Engineering oilseeds to produce substantial amounts of FAs synthesized in this way is a challenge that still has not been overcome. For example, transfer of a gene encoding an oleate hydroxylase from castor bean to the model oilseed *Arabidopsis* has resulted in seed oil containing only 17 -20% hydroxy FAs. Castor bean produces oil in which nearly 90% of the FAs in the seed oil contain a hydroxy group. Similar results have been observed in plants engineered to produce epoxy, acetylenic and various conjugated FAs. Over the last 10 years, progress in this area has regularly been reported in review papers [e.g., 33, 34]. For this reason, the current review will be restricted to a general over-view of the subject. It is important to note that considerable progress has been made, and the study of the assembly of

TAG with unusual FAs has contributed substantially to the understanding of plant cell biology and lipid metabolism.

The majority of the enzymes catalyzing the modification of FAs on PC belong to the *FAD2* gene family and are similar in sequence and structure to the *FAD2* plant extra-plastidial oleate desaturase, an enzyme that catalyzes the insertion of a double bond between carbons 12 and 13 of oleate to produce linoleate [<http://www.annualreviews.org/doi/abs/10.1146/annurev.-arplant.49.1.611?journalCode=arplant.2>]. Examples include the FA hydroxylase from castor (CFAH12) which acts on oleoyl moieties esterified to the *sn*-2 position of PC and introduces a hydroxy group on carbon 12. Divergent *FAD2*s from calendula and tung that act on the $\Delta 9$ or $\Delta 12$ double bonds of linoleoyl moieties esterified to PC to produce FAs with conjugated double bond systems. Expression of such enzymes in oilseeds, such as Arabidopsis, results in low levels of accumulation of the expected FA, and is generally characterized by a significant increase in levels of oleic acid and reduction of PUFA content. In addition, higher levels of unusual FAs often correlate with reduced oil content, shrivelled seeds and problems with germination [<http://ddr.nal.usda.gov/bitstream/10113/32285/1/IND43818702.pdf>; <http://www.botany.ubc.ca/kunst/Journals/L.%20lindheimeri.pdf>].

A general consensus now exists that the failure to achieve high level production of unusual FAs is due to multiple bottlenecks in the process of unusual FA synthesis and their incorporation into TAG. To understand this, research has focused on the detailed characterization of plants that naturally make the target FAs, and on plants engineered to do so. In terms of the modifying enzyme, protein expression levels, stability and turnover, and the availability of electron transport cofactors have been implicated as potentially important issues. In castor, for

example, the CFAH12 protein is highly represented in seed-derived EST sequence collections and is a highly abundant protein in the ER, as judged from proteomic analysis [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1950504/pdf/1471-2229-7-42.pdf>; <http://www.ncbi.nlm.nih.gov/pubmed/11870775>]. It is not yet clear whether equivalent levels of protein production have been achieved, or are even required in transgenic plants. Transcriptional or post-translational regulation of FAD2-type enzymes is also poorly understood.

A notable bottleneck in transgenic seeds appears to be in the removal of the unusual FAs from their site of synthesis. In the native plant, the newly formed unusual FAs are rapidly removed from PC and used for TAG assembly, whereas in engineered oilseeds, accumulation of the unusual FAs in membrane lipids has been observed, likely with detrimental effects on seed development. How plants maintain the FA composition of their membranes is still an area of debate. *In vitro* studies using microsomal membrane preparations from castor and safflower suggested that an endogenous PLA₂ activity, releasing free FAs, was responsible for removal of ricinoleic acid from PC [35]. This has prompted a search for candidate PLA₂ enzymes in plants that make unusual FAs, and also for acyl-activating enzymes that would be required to generate the acyl-CoA required for TAG assembly by the Kennedy pathway. Alternative mechanisms for removal of unusual FAs from PC have been postulated including the reverse reaction of LPCAT and activities catalyzed by PDAT and the recently discovered PDCT [10]. To date the relative role of such enzymes is not clear.

A further problem appears to be in the utilization of unusual FAs by the endogenous acyltransferases of TAG assembly, with the preferences of the enzymes being for the native FAs over the new substrates, thus limiting incorporation into TAG. Recent promising breakthroughs

include the discovery of acyltransferases that preferentially incorporate unusual FAs into TAG. DGAT2s from castor bean and *Vernonia galamensis*, for example, have been shown to increase incorporation of unusual FAs into the *sn*-3 position of TAGs in transgenic plants when co-expressed with the appropriate enzymes for biosynthesis of said unusual FAs (<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2908398/pdf/nihms217608.pdf>; <http://onlinelibrary.wiley.com/doi/10.1111/j.1467-7652.2009.00476.x/abstract>].

There is an increasing body of evidence that suggests that in plants such as castor, enzymes of TAG biosynthesis may have co-evolved with enzymes of unusual FA biosynthesis, resulting in almost exclusive production and storage of the unusual FA in the seed oil. The engineering of high levels of unusual FAs in transgenic oilseeds will therefore likely require whole pathway engineering rather than single gene transfer. This may require down-regulation of endogenous enzymes in addition to the transformation of the target plants with new enzymes of FA biosynthesis and TAG assembly.

The efficient breakdown of these novel FAs during seed germination and seedling establishment is another area which will require further scrutiny. Engineered plants will need to be able to utilize these lipids for growth and development, otherwise germination will be adversely affected.

256.10 INCREASING SEED OIL CONTENT AND PRODUCING OIL IN VEGETATIVE TISSUE

Increased seed oil content is important for maintaining an adequate supply of oils for both food and non-food applications. This section of the review examines manipulation of enzyme targets in carbon flow and the role of transcription factors (TFs) in regulating embryo

development and TAG accumulation. We then demonstrate how molecular insights into oil accumulation in the seed are providing tools for inducing oil accumulation in plant tissues other than seeds or fruit.

256.10.1 Targeting Specific Enzyme-Catalyzed Reactions in Carbon Flow

A number of studies have demonstrated increases in seed oil content brought about through manipulation of single enzyme-catalyzed reactions involved in lipid biosynthesis and other aspects of primary metabolism [19, 36, 37]. Most investigations are based on ‘proof of concept’ work with *Arabidopsis*. In some cases, studies were extended to major crops such as soybean and *B. napus*. We will only discuss a few examples in this review.

In an early investigation, a relative seed oil content increase of about 5% was achieved in *B. napus* by engineering the plant to produce *Arabidopsis* cytosolic ACCase in the *B. napus* plastid [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC158117/pdf/1130075.pdf>]. Presumably, the cytosolic enzyme was effective in increasing seed oil content because it was not under the same regulatory control as the plastidial form of the enzyme.

LPAAT was the first enzyme in TAG assembly to be explored for increasing seed oil content. A mutated form of a gene encoding *sn*-2 acyltransferase from yeast was used to transform both *Arabidopsis* [<http://www.plantcell.org/cgi/reprint/9/6/909.pdf>] and *B. napus* [38]. Field studies with transgenic HEAR indicated a relative increase in seed oil content of up to 13.5%. Recently, two *LPAAT* homoeologs from *B. napus* were used to transform *Arabidopsis* [<http://www.plantphysiol.org/cgi/content/abstract/152/2/670>]. Expression of each cDNA resulted in seeds with increased oil content and overall mass.

DGAT, which catalyzes the acylation of *sn*-1,2-DAG to produce TAG (Figure 3), has received considerable attention as target for increasing seed oil content. Two types of membrane-bound DGAT (DGAT1 and DGAT2) sharing no homology have been identified in various plants whereas a soluble DGAT was purified and cloned from developing peanut (*Arachis hypogea*) cotyledons [38; <http://www.jbc.org/content/276/42/38862.full.pdf+html> ;<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1533943/pdf/pp1411533.pdf>]. Recently, Rani et al. identified another soluble DGAT, associated with surface cutin formation in Arabidopsis, with broad specificity for substrates containing non-hydroxy, mono and dihydroxy FAs [<http://www.jbc.org/content/early/2010/10/04/jbc.M110.133116.long>]. The investigators suggested that this DGAT may generate a pool of TAG to provide FAs for cutin synthesis. An Arabidopsis wax synthase, displaying some DGAT activity, has also been identified [<http://www.plantphysiol.org/cgi/reprint/148/1/97>]. Hydropathy plot analysis indicates that DGAT1 has considerably more transmembrane segments than DGAT2. The soluble DGAT from peanut shares a low level of identity with DGAT1 or DGAT2. DGAT2 proteins from certain plant species producing industrially-useful FAs, including ricinoleic, eleostearic and vernolic acid, have been shown to exhibit increased preference for their respective unusual FAs [40; <http://www.plantcell.org/cgi/reprint/18/9/2294>; <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2908398/pdf/nihms-217608.pdf>; <http://onlinelibrary.wiley.com/doi/10.1111/j.1467-7652.2009.00476.x/abstract>]. Therefore, cDNAs encoding these DGAT2 proteins may be useful in engineering major oilseed crops to produce unusual FAs for industrial applications. Several lines of evidence have suggested that the level of DGAT activity may have a substantial effect on the flow of carbon into seed oil [36]. Subsequently, several investigations have focused on the over-expression of *DGAT* cDNAs in various oilseeds. Seed specific over-expression of an Arabidopsis *DGAT1* in

Arabidopsis resulted in relative increases in seed oil content of up to 28% [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC111175/pdf/pp000861.pdf>]. The first published report on over-expression of *DGAT1* as a means of increasing seed oil content in a major oilseed crop was based on the expression of Arabidopsis *DGAT1* or *B. napus DGAT1* in *B. napus* [http://www.ispl2006.msu.edu/Proceedings_Book.pdf]. Top-down control analysis of FA biosynthesis (Block A) and TAG assembly (Block B) in developing seeds of *B. napus* indicated that 70% of the control in overall TAG accumulation resided with Block B reactions [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2561151/pdf/ern206.pdf>]. In contrast, transgenic *B. napus* over-expressing *DGAT1* exhibited a drop in control to about 50% in Block B which was probably attributable to increased DGAT activity in the developing embryo. Thus, flux analysis may be a useful tool in developing metabolic engineering strategies. The same publication demonstrated that over-expression of *DGAT1* in *B. napus* also reduced the penalty on seed oil content caused by drought. A recent study on antisense suppression of *DGAT1* in *B. napus* indicated a decreased seed oil content which was compensated by an increase in seed protein content [<http://www.ncbi.nlm.nih.gov/pubmed/19602173>]. Microarrays have been used to investigate the transcriptome in *B. napus* over-expressing Arabidopsis *DGAT1* [<http://www.biomedcentral.com/content/pdf/1471-2164-9-619.pdf>]. Transcripts encoding enzymes in the G3P pathway, leading to TAG (see Figure 3), were up-regulated, but there were also other transcriptional changes which may be related to feedback or feed-forward effects caused by the increase in DGAT1 activity. Heterologous expression of a codon-optimized form of a fungal *DGAT2A* in soybean (*Glycine max*) has also been shown to increase seed oil content [<http://www.plantphysiol.org/cgi/reprint/148/1/89>]. Resulting transgenic lines exhibited a statistically significant absolute increase in seed oil content of about 1.5% under both greenhouse and field

conditions. Recently, a high throughput yeast-based system designed to screen for recombinant DGAT variants with enhanced catalytic efficiency was developed [<http://www.springerlink.com/content/b578131234257327/>]. Thus, high performance DGATs developed through directed evolution could potentially be useful in increasing seed oil content to even greater levels than currently achieved.

Oil accumulation can also be affected by reactions in carbon flow which provide building blocks for lipid biosynthesis. The mitochondrial pyruvate dehydrogenase complex catalyzes the production of acetyl-CoA and carbon dioxide from pyruvate and CoA, and represents a connection between glycolysis and the citric acid (TCA) cycle. Mitochondrial pyruvate dehydrogenase kinase is known to down-regulate the pyruvate dehydrogenase complex by phosphorylation. Antisense suppression of the gene encoding this kinase has been shown to result in increased seed oil content and seed weight in Arabidopsis [<http://www.springerlink.com/content/j416416774m15613/>; <http://jxb.oxfordjournals.org/content/54/381/259.full.pdf+html>]. Feeding studies with radioactively-labeled pyruvate supported the hypothesis that the observed increase in seed oil content was associated with an increased supply of acetyl-CoA from the mitochondria.

In a second example, disruption of cytosolic glucose-6-phosphate dehydrogenase in Arabidopsis was also shown to lead to increased seed oil content and increased seed weight [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2230552/pdf/pp1460277.pdf>]. Operating within the oxidative pentose phosphate pathway, glucose-6-phosphate dehydrogenase catalyzes the conversion of D- glucose-6-phosphate dehydrogenase and NADP⁺ into D-glucono-1, 5-lactone 6-phosphate and NADPH. The observed increase in seed oil content was brought about by a

decrease in precursors for oil production rather than a decrease in NADPH supply for FA biosynthesis.

256.10.2 Altering the Action of Transcription Factors

Transcriptional regulation is an important step in the control of seed oil accumulation [36, 37; <http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2008.03461.x/full>]. For example, transcript levels of genes involved in lipid biosynthesis are coordinated during seed development [37; <http://www.ncbi.nlm.nih.gov/pubmed/19463193>] and, for several enzymes in the pathway, transcript abundance is highly correlated with enzymatic activity [<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2006.02682.x/full>]. Importantly, genetic and transgenic studies have shown that altering levels of certain TFs can have profound effects on the expression of multiple genes implicated in lipid biosynthesis with corresponding changes in seed oil composition and /or content. Genetic engineering of individual TFs has thus been proposed as an effective strategy for modulating the expression of several genes within the lipid biosynthetic pathway. Genetic and biochemical analyses of TFs involved in controlling seed oil deposition are consistent with the existence of hierarchical transcriptional cascades, in which specific transcription factors are responsible for regulating the expression of a series of genes, some of which may encode other transcription factors, which in turn regulate the expression of other gene sets. In theory, TFs near the top of the hierarchy are typically responsible for controlling broad developmental and metabolic processes, while those near the bottom may regulate specific biochemical steps within a pathway. Thus, it is critical that the TFs selected for metabolic engineering provide selective control of relevant pathways with minimal pleiotropic effects.

WRINKLE1 (WRI1) currently represents the best candidate TF for genetic engineering of seed oil content. As its name implies, WRI1 was identified as an Arabidopsis loss-of-function mutant producing wrinkled seeds [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC34877/>]. This phenotype is associated with a dramatic reduction (80%) in seed oil content and an increase in starch accumulation. Conversely, constitutive overexpression of *WRI1* using the cauliflower mosaic virus 35S promoter (pCaMV35S) in wild-type Arabidopsis resulted in a 10-20% increase in seed oil levels [<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2004.02235.x/full>]. Transgenic overexpression has also been reported with *WRI1* genes isolated from two other plant species. Expression of either of two *WRI1* genes from *B. napus* in transgenic Arabidopsis increased seed oil content by up to 20% with no change in protein levels [<http://www.ncbi.nlm.nih.gov/pubmed/19828328>]. Although the effectiveness of the Brassica genes was not tested in transgenic *B. napus*, endogenous expression levels of these genes were correlated with seed oil content in different genotypes, and a *WRI1* polymorphism was shown to be significantly associated with oil content in a segregating population. Our group has performed a complementary experiment by expressing the Arabidopsis *WRI1* in transgenic *B. napus* and observed increases in seed oil content with no changes in FA composition (N. Sharma and P. Fobert, unpublished). In a detailed study that included replicated, multi-site field testing of elite hybrids, transgenic expression of a maize *WRI1* homolog from an embryo-specific promoter was shown to increased oil content in grains by 10-22%, with a concomitant reduction in starch levels (60%), but no change in protein content [<http://www.plantphysiol.org/cgi/content/full/-153/3/980>]. Transgenic expression of *Zea mays* *WRI1* was not associated with any notable effects on plant development, including germination, flowering time, or grain weight. The results from the maize study provide the first rigorous testing of the *WRI1* gene outside of laboratory

settings and strongly suggest that it may be a commercially-relevant target for seed oil improvement.

Although the maize study did not detect changes in grain size, expression of Brassica *WRII* in Arabidopsis resulted in substantial increases in seed weight (up to 40%), which were attributed to bigger embryos consisting of larger cells [<http://www.ncbi.nlm.nih.gov/pubmed-/19828328>]. The sizes of siliques and the number of seeds per silique were not affected. Expression of Arabidopsis *WRII* in Arabidopsis did not affect the number of seeds produced per plant but possible effects on seed size were not reported [<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2004.02235.x/full>]. In contrast to the above studies, Baud et al. [41] reported serious pleiotropic effects associated with expression of Arabidopsis *WRII* driven by a dual pCaMV35S promoter, ranging from stunted growth and reduced apical dominance, to poor seed set, reduced seed size and reduced seed oil content. Expression of Arabidopsis *WRII* using a seed-specific promoter alleviated these effects, but did not lead to increases in seed oil content, despite 25-70 fold higher steady-state levels of *WRII* mRNA. The reason for these differences in results between studies remains to be resolved, although Baud et al. [41] discuss possibilities including choice of promoter, ecotype, and differences in growth conditions. Previous work by these authors highlighted the importance of expression patterns to *WRII* function [<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2007.03092.x/full>]. It is also noteworthy that ectopic expression of *WRII* in vegetative tissue can lead to TAG synthesis in seedlings which negatively impacts germination performance [<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2004.02235.x/full> ; <http://www.plantphysiol.org/cgi/content-full/141/2/745>].

WRI1 belongs to the plant-specific APETALA2 (AP2) family of TFs [<http://online-library.wiley.com/doi/10.1111/j.1365-313X.2004.02235.x/full>]. It likely regulates oil biosynthesis by binding to conserved sequences within the promoters of genes encoding enzymes involved in late glycolysis and FA biosynthesis, activating their transcription. Steady-state mRNA levels of for these genes are reduced in *wri1* mutants but increased in transgenic seeds overexpressing *WRI1* and in seedlings ectopically expressing *WRI1* [<http://online-library.wiley.com/doi/10.1111/j.1365-313X.2007.03092.x/full>; <http://www.plantphysiol.org/cgi/content/full/153/3/980>; 41, 42]. Correspondingly, the activities of several glycolytic enzymes are compromised in developing *wri1* seeds [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC34877/>] and flux through plastidial pyruvate kinase, which provides most of the carbon for FA biosynthesis, is dramatically reduced [[http://www.plantphysiol.org/cgi/content-full/151/3/1617](http://www.plantphysiol.org/cgi/content/full/151/3/1617)]. WRI1 binds *in vitro* to a conserved sequence called the AW-box found in the promoter of many of the genes differentially regulated in the mutant or transgenic overexpressing lines, and is capable of trans-activating the expression of a reporter gene fused to these promoters in transient assays [42]. Although *DGAT1* mRNA levels are reduced in the *wri1* mutant, levels of *DGAT1* mRNA do not change in seedlings ectopically expressing *WRI1*, nor is WRI1 capable of *trans*-activating from the *DGAT1* promoter [42]. Similarly, transcripts levels of *DGAT1*, *GPAT* and *oleosin* were unaffected by increased *Z. mays* *WRI1* expression in maize embryos [<http://www.plantphysiol.org/cgi/content/full/153/3/980>]. Interestingly, targeted expression of *Z. mays* *WRI1* in the maize endosperm did not result in increased oil accumulation, presumably because genes for TAG assembly and oil body formation are not expressed in this tissue [<http://www.plantphysiol.org/cgi/content/full/153/3/980>]. Together, these data indicate that WRI1 does not regulate genes involved directly in TAG assembly [42].

One level above WRI1 in the transcriptional hierarchy is LEAFY COTYLEDON2 (LEC2), a member of the B3 family of TFs. B3 TFs bind to the conserved RY element found in the promoter of many genes expressed during seed maturation. Similar to WRI1, Arabidopsis *lec2* mutants have reduced seed oil content while ectopic expression of *LEC2* activates genes involved in oil biosynthesis resulting in the accumulation of storage lipids [reviewed in <http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2008.03461.x/full>]. Consistent with the notion that WRI1 is a target of LEC2, steady-state levels of *WRI1* mRNA increase in seedlings following activation of inducible versions of LEC2. LEC2-induced synthesis of storage lipids in seedlings is not observed in the *wri1* mutant, indicating that WRI1 is an important mediator of LEC2 function. However, ectopic expression of *WRI1* in a *lec2* mutant background is not sufficient to rescue the *lec2* phenotype, suggesting that other mediators of LEC2 also exist [<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2007.03092.x/full>].

Unlike WRI1, altering LEC2 levels has dramatic consequences on embryogenesis and seed maturation which are consistent with the role of a master regulator for these processes. To this end, LEC2 functions in concert with other two other B3 TFs, ABSCISIC ACID INSENSITIVE3 (ABI3) and FUSCA3 (FUS3) as well as another leafy cotyledon TF (LEAFY COTYLEDON1 or LEC1) that is structurally unrelated to the B3 family [<http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2008.03461.x/full>]. Seed oil biosynthesis and seed storage protein accumulation represent only a subset of the processes controlled by LEC1, LEC2, ABI3 and FUS3 during seed maturation. As described for LEC2, LEC1-induced storage lipid synthesis in seedlings also requires WRI1 [<http://www.plantphysiol.org/cgi/content/full/148/2/1042>]. Since induction of LEC1 in seedlings did not activate *LEC2* expression, it was postulated that LEC1 and LEC2 may control two

parallel pathways towards WRI1 activation. *Z. mays* LEC1 is also capable of *trans*-activating a *Z. mays* *WRI1* promoter fusion in transient assays, further suggesting that WRI1 is a target (direct or indirect) of LEC1 [<http://www.plantphysiol.org/cgi/content/full/153/3/980>]. The interactions between WRI1 and ABI3 and FUS3 have not been clearly defined. ABI3 and WRI1 were suggested to act independently in regulating germination [<http://www.plantphysiol.org/cgi/content/full/141/2/745>], but the interaction of these TFs during seed maturation needs to be further explored.

In summary, LEC1, LEC2, ABI3 and FUS3 are important regulators of seed oil biosynthesis that function by modulating WRI1. However, the pleiotropic effects associated with overexpression of these TFs make them poor targets for genetic engineering of seed oil accumulation at present. This was convincingly shown in maize, where overexpression of *Z. mays* *LEC1* was associated with reduced germination and vegetative growth [<http://www.plantphysiol.org/cgi/content/full/153/3/980>].

Given that LEC1, LEC2, ABI3 and FUS3 are strong positive regulators of seed maturation, mechanisms have evolved to ensure that their expression is suppressed during vegetative growth. TFs contributing to this suppression have been identified as loss-of-function mutants that ectopically express master regulators outside of the seed. Although the genes encoding these suppressors are unlikely to be of direct interest for enhancing seed oil content, they could find applications towards the metabolic engineering of storage lipids in vegetative tissues such as leaves, tubers and roots (see section 256.10.3). These applications may result in higher energy index in forage crops.

Suppressors of seed maturation programs in vegetative tissues are exemplified by *PICKLE* (*PKL*), which encodes a putative chromatin re-modeling factor belonging to the chromodomain/helicase/DNA-binding (CHD3) family [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC24151/>]. Steady-state levels of *LEC1*, *LEC2* and *FUS3* mRNA are close to 1000-fold higher in roots of *pk1* mutants than the wild-type plant, leading to the ectopic expression of embryogenic genes, the synthesis of storage lipids and swollen green primary root tips [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2515612/>]. Mutation of the two closest relatives of PKL in Arabidopsis (*PKR1* and *PKR2*) does not yield a *pk1* phenotype; however, the *pk1* phenotype is more penetrant in the *pk1 pk2* double mutant, indicating a partially redundant function [<http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1000605>].

CHD3 proteins are known to act as transcriptional repressors, often as part of complexes possessing histone de-acetylase activity. However, there is currently no evidence to support direct binding of PKL to the promoters of genes ectopically activated in the *pk1* mutant [<http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1000605>], nor does the histone acetylation status of chromatin appear to be altered in the mutant [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2504882/>]. Instead, PKL has been proposed to transcriptionally activate its direct targets, which include a subset of Polycomb group proteins [<http://www.plosgenetics.org/article/info%3Adoi%2F10.1371%2Fjournal.pgen.1000605>] which are involved in gene silencing by imparting repressive chromatin marks upon target genes, specifically trimethylated histone H3 lysine 27 (H3K27m3). Consistent with this notion, levels of H3K27m3 are reduced in the promoters of genes differentially regulated in the *pk1* mutant, including *LEC1*, *LEC2*, *ABI3* and the Polycomb group protein genes *EMF2* and *SWN*. It is

proposed that repression of *LEC1*, *LEC2*, *ABI3* and *FUS3* in vegetative tissues by PKL is indirect, and mediated by Polycomb group proteins.

Plants encode a clade group of B3 TFs, distinct from *ABI3*, *FUS3* and *LEC2*, which act redundantly to suppress the expression of seed maturation programs in vegetative tissues [<http://www.plantphysiol.org/cgi/content/full/143/2/902>; <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1785360/>]. In addition to the B3 domain, these B3 factors contain domains associated with chromatin re-modeling factors and transcriptional repressors. Double mutants in two of these factors (*HIGH-LEVEL EXPRESSION OF SUCROSE INDUCIBLE GENE2* (*HSI2*) – also known as *VAL1* – and *HIS2-like* (*HSIL* or *VAL2*) show seedling expression of *LEC1*, *ABI3*, *FUS3*, *LEC2* and large numbers of embryo-specific genes containing RY elements in the promoters, the production of storage compounds and ectopic embryogenesis. Both the *pkl* and *hsi2* phenotypes are more pronounced after treatment with inhibitors of gibberellic acid biosynthesis, implicating this growth regulator in the suppression of seed maturation programs in vegetative tissues.

ASIL1 represents another suppressor of B3 master regulators that accumulates storage lipids in seedling tissues upon mutation [<http://www.plantcell.org/cgi/content/full/21/1/54>]. Although it displays a number of developmental abnormalities, the *asil1* mutant does not produce *pkl* roots or ectopic embryos reminiscent of the *pkl* or *val* mutants.

In addition to the TFs mentioned above, several others have been shown to be implicated directly or indirectly in the regulation of seed oil biosynthesis [reviewed in 36, 37; <http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2008.03461.x/full>]. For the most part, analysis of these regulators has been limited to work in *Arabidopsis* and their mechanisms of

action remain to be elucidated. Of note is a recent study in confirming that *GLABRA2* genes from *B. napus* act as negative regulators of seed oil synthesis when expressed in Arabidopsis

[<http://www.springerlink.com/content/g002806408x666u2/>]. The report also identified molecular markers for different members of the *GLABRA2* gene family in Brassica, which could be exploited in marker assisted breeding, although it remains to be determined whether natural variation in *GLABRA2* genes contribute to seed oil content in this or other crop species.

256.10.3 Production of Triacylglycerol in Vegetative and Root Tissue

The investigation of various molecular strategies to increase seed oil content has resulted in novel approaches to increase the oil content of vegetative tissue which normally produces low levels of TAG. The production of TAG in leaves could open new opportunities for biofuel production which would not interfere with the supply of food oil coming from oleaginous seeds or fruits [19]. The TAG content of leaves of several plant species was surveyed by Lin and Oliver [43]. Labeling studies with ^{14}C -acetate suggested that leaf TAG, along with carbohydrate, served as a diurnal photosynthetic store. The TAGs were present as oil bodies in the cytoplasm and were presumably derived from the ER. Previously, constitutive over-expression of Arabidopsis *DGAT1* in tobacco (*Nicotiana tabacum* L. cv Xanthi) leaves was shown to result in a 7-fold increase in TAG content. As described in section 256.10.2, ectopic expression of *LEC2* and other B3 TFs in Arabidopsis leaves under the control of an inducible promoter was shown to lead to production of seed-specific TAG in leaves [[http://onlinelibrary.wiley.com/doi/10.1111-j.1365-313X.2008.03461.x/full](http://onlinelibrary.wiley.com/doi/10.1111/j.1365-313X.2008.03461.x/full)]. Recently, Slocombe et al. [44] reported increased TAG production in Arabidopsis leaves by blocking FA oxidation or through ectopic expression of *LEC2* in the FA breakdown mutant *comatose* (*cts2*). Andrianov et al. [45] achieved a 20-fold

increase in the TAG content of tobacco (*N. tabacum* L. cv Wisconsin) leaves through expression of Arabidopsis *DGAT1* under the control of a strong ribulose-biphosphate carboxylase small subunit promoter. TAG constituted up to 5.8% of the dry biomass in cultivar Wisconsin and up to 6% of the dry biomass in a high-sugar tobacco variety (NC-55). Previously, it was demonstrated that increased sucrose concentration in the medium of *B. napus* microspore-derived cell suspension cultures leads to a transient increase in DGAT activity and TAG content [46]. Adrianov et al. [45] also expressed Arabidopsis *LEC2* in tobacco leaves under the control of an inducible promoter and achieved TAG accumulation levels of up to 6.8% of the dry biomass.

Recently researchers in New Zealand have co-expressed a gene encoding polyoleosin (two or more oleosin repeat units) with *DGAT1* in vegetative plant tissue, allowing the generation of emulsion complexes containing only the desired species of oleosin (since oleosins are not normally expressed in the vegetative portions of plants). This resulted in novel oil bodies encapsulated (and protected from degradation) by the polyoleosin in vegetative tissues. In forage grasses, this may result in increased digestible energy for livestock. More importantly, it will expand the types of plant tissues capable of oil production for biofuels beyond the seed alone [<http://www.faqs.org/patents/app/20090133160>]. In another new discovery, the disruption of the gene encoding the human homolog of CGI-58 in Arabidopsis was shown to result in accumulation of lipid droplets in the cytosol [<http://www.ispl2010.org/-program.cfm?progday=Tuesday>]. In mammalian systems, CGI-58 appears to facilitate lipolysis on lipid droplets and a defect in the human gene results in a disorder (Chanarin-Dorfman syndrome) characterized by abnormal intracellular accumulation of lipid droplets in various

tissues [<http://www.jlr.org/content/48/5/1078.full.pdf+html>]. Thus, there appear numerous approaches whereby storage lipid biosynthesis can be encouraged to occur in vegetative tissue.

TAG has also been shown to accumulate to substantial levels in the stem tissue of *Tetraena mongolica* Maxim [http://www.labpmp.cn/news/lunwen_file/20071126132243.pdf]. This plant is often referred to as ‘oil firewood’. The phloem cells of these stems are enriched in cytoplasmic lipid bodies (oleosomes) and contain about 90 mg of TAG per g of dry matter. Since the plant hardly sets seeds, the stem is believed to have evolved as a carbon storage organ to support the growth of new shoots later in the year.

TAG accumulation has also been observed in chloroplasts in leaves [reviewed in 8]. TAG accumulation has been investigated during leaf senescence in *Arabidopsis* [<http://www.plantphysiol.org/cgi/reprint/129/4/1616>]. *DGAT1* transcript and DGAT1 protein (immunochemically detected) increased with TAG levels during senescence. The plastidial DGAT1, however, had a molecular mass of 29 kDa which was considerably smaller than the molecular mass of 51 kDa deduced from the encoding cDNA. It was suggested that the temporary incorporation of FAs into TAG during senescence allowed membrane degradation to progress without a build-up of deleterious free FAs.

Substantial TAG accumulation can also occur in roots. Yellow nutsedge (*Manihot esculenta*) naturally accumulates about 25% oil and 30% starch in the tubers and may represent a useful model plant system for deciphering mechanisms that govern the flow of carbon into oil *versus* starch [22]. TAG accumulation in roots can also be induced. In addition, the *pk1* mutant of *Arabidopsis* has been shown to accumulate TAG with FA composition similar to seed oil [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2536513/pdf/nihms-58142.pdf>].

256.11 PLATFORM CROPS FOR INDUSTRIAL OIL PRODUCTION

As suggested previously in this review, it is imperative that an increase in the utilization of plant oil for non-food applications does not compromise the supply or quality of oil destined for human consumption. This is a complex and sensitive issue that must be addressed as a priority. Opinions vary as to how to approach this problem.

One possible solution being explored is the development of oilseed crops dedicated for the production of industrial oils. This can entail the domestication of plant species that naturally make oil of the desired composition (Table 4), or the development of “platform crops”- existing oilseed crop species engineered to synthesize oil only for industrial use. A recent review describes potential platform crops under consideration largely for temperate climate industrial oil production [22]. Two species of particular interest are *B. carinata* and *Camelina sativa*. These oilseeds are not currently grown on a large scale, but already have yield and agronomic characteristics that make them suitable for commercial production using conventional farming practices. Both are easy to transform and seed oil profile manipulation has been demonstrated. In addition, they are more tolerant of drought and have lower nutrient requirements compared to existing temperate oilseed crops. These species can be grown in areas where oilseed crops currently do not do well, thus expanding the area available for oilseed production and introducing new crop rotation options for farmers.

Production of industrial crops, particularly those with FA composition less suitable for human consumption, will require carefully managed identity preservation systems that operate from field to final product. This will include management of crop in production, and of volunteers in subsequent crops. Robust identity preservation systems are already successfully

applied for crops such as HEAR *B. napus* and malting varieties of barley. The platform crop approach is attractive because it offers the opportunity for a clear separation of food crops and industrial crops.

256.12 POTENTIAL FOR WAX-BASED INDUSTRIAL LIPIDS

The global market for natural and synthetic waxes was estimated at close to US\$7.9 billion in 2008 with an annual growth rate of nearly 5%. Petroleum wax, derived from crude oil, is the most common type of wax currently in use. Natural waxes such as the liquid wax from jojoba seeds and the solid waxes collected from the leaf surfaces of the Carnuba palm (*Copernicia cerifera*) and desert shrubs including *Euphorbia cerifera* and *Euphorbia antisiphilitica* (candelilla wax) tend only to be used in specialized, high value applications. Cosmetics and toiletries manufacture, for example, accounts for over 90% of jojoba oil consumption. Plant derived liquid and solid waxes differ in chemical composition, with the solid waxes tending to be a complex mixture of hydrocarbons whereas jojoba oil is predominantly composed of wax ester (Figure 1). A comprehensive review of natural waxes is available in Ullmann's Encyclopedia of Industrial Chemistry [<http://onlinelibrary.wiley.com/doi/10.1002/14356007.a28103/full>].

In the wax formation pathway in jojoba, cytoplasmic oleoyl-CoA (derived from plastidial-produced oleic acid) can be elongated to form eicosenoyl-, erucoyl- and nervonoyl-CoA. Fatty alcohols derived from these FAs are produced through the catalytic action of acyl-CoA reductase [<http://www.ncbi.nlm.nih.gov/pmc/articles/PMC161098/pdf/080281.pdf>; <http://www.plantphysiol.org/cgi/reprint/122/3/635>]. In turn, elongated acyl-CoAs and fatty alcohols are used as co-substrates in the synthesis of linear esters catalyzed by wax synthases

(fatty acyl-CoA:fatty alcohol acyltransferase) [<http://www.plantphysiol.org/cgi/reprint-/122/3/645>]. As indicated previously in section 256.10.1, a cDNA was identified which encoded an Arabidopsis wax synthase with some DGAT activity [<http://www.plantphysiol.org/cgi/reprint-/148/1/97>]. The recombinant enzyme, when produced in *E. coli*, exhibited DGAT activity which was 10-fold less than wax synthase activity. The true catalytic focus between oil/wax formation, as encoded by the many putative DGAT/WS genes, remains to be determined.

Due the excellent lubricity and stability of liquid waxes in lubricant applications, there has been considerable interest in engineering wax ester production in commercial oilseed crops.. Expression of the cDNAs encoding jojoba wax synthase and acyl-CoA reductase together with the cDNA encoding KCS in *B. napus* led to the successful diversion of FAs from the TAG assembly pathway to the newly engineered wax ester pathway. Although the resulting seed oil contained up to 49% wax esters, commercial production has not been reported [47]. This work, pioneered by the biotech company Calgene during the early 1990s, is outlined in US patent number 5,723,747 [<http://www.freepatentsonline.com/5723747.html>]. A multinational research project to develop the production of novel wax esters in non-food oilseed crops has recently been approved for funding by the European commission as part of the Seventh Framework Programme [<http://icon.slu.se/ICON/index.htm>]. In addition to characterizing the pathways of wax ester biosynthesis, plant surface waxes and their biosynthetic pathways have been under intense scrutiny with the goal of engineering new chemical feedstocks and biofuels [<http://www-botany.ubc.ca/kunst/Journals/Biofuels.pdf>].

256.13 CLOSING COMMENTS

It is clear that with the current state of knowledge, including ever-increasing lipid-related gene discovery and characterization, as well as ease of transformation of target platform crops and “proof of concept” experiments including confined field trials of transgenic prototypes which show the power of transgene technologies, that the future bodes well for creating new and industrial feedstock oils and biofuels. These will have the advantages of having a smaller carbon footprint compared to fossil fuels and enhancing agricultural and environmental sustainability. Despite these advantages, clearly there are several hurdles to overcome: The cost issue is always before us and there is currently an economic *disadvantage* in trying to compete with fossil fuels. Identity preservation of industrial plant oilseeds and isolation of these from the food stream will require contract growers and associated specialized crushing facilities and marketing strategies. Homogeneity of the transgenic plant oil products is another bottleneck that needs to be addressed. Currently many of the factors which ensure 90% ricinoleic acid content in castor bean oil or 75% erucic acid content in nasturtium seed oil are not fully understood. Thus, we will need to expand our knowledge, not only of the biochemistry of lipid biosynthesis, but also our insight into the regulation, both transcriptionally and translationally, of genes and gene products, respectively. Despite these challenges, these are tractable problems and with the increased industrial interest and pressure as planetary stewards to produce “green” biofuels and chemical feedstocks, we will no doubt reach these goals over the next decade.

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Figure Legends

Figure 1. Chemical structures of plant storage lipids.

Figure 2. Chemical structures of plant fatty acids. PUFA, polyunsaturated fatty acid; VLCFA, very long chain fatty acid.

Figure 3. Diagram showing the intersecting biochemical pathways for fatty acid and glycerolipid biosynthesis in oilseed cotyledons of the *Brassicaceae*. Also shown is the effect of expressing in Brassica a cDNA encoding $\Delta 5$ fatty acyl-Coenzyme A desaturase (DES 5) from *Limnanthes* sp.. 3-Ketoacyl-CoA synthases (KCSs) are the elongase enzymes which catalyze the first step in a four-enzyme fatty acid elongase complex which produces the very long chain fatty acids. Fatty acid desaturase (FAD)2 and FAD3 are 18:1 $\Delta 9$ *cis* and 18:2 $\Delta 9$ *cis* desaturases catalyzing the formation of 18:2 $\Delta 9, 12$ *cis* and 18:3 $\Delta 9, 12, 15$ *cis*, respectively. Other enzymes: ACoASase, acyl-Coenzyme A synthetase; CPTase, cholinephosphotransferase; DGAT, diacylglycerol acyltransferase; FAD, fatty acid desaturase; FAT, fatty acid thioesterase; GPAT, *sn*-glycerol-3-phosphate acyltransferase; KAS, β -ketoacyl-acyl carrier protein synthase; KCS, 3-ketoacyl-Coenzyme A synthase; LPAT (LPAAT), lysophosphatidic acid acyltransferase; LPCAT, lysophosphatidylcholine acyltransferase; PAPase, phosphatidic acid phosphatase; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; SAD, saturated acyl-Coenzyme A desaturase; SLC1-1, *Saccharomyces cerevisiae* lysophosphatidic acid acyltransferase. Other abbreviations: ACP, acyl carrier protein; CoA, Coenzyme A; DAG, diacylglycerol; FAS, fatty acid synthesis; G3P, *sn*-glycerol-3-phosphate; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; Mal-CoA, malonyl-CoA; PA, phosphatidic acid; PC, phosphatidylcholine; TAG, triacylglycerol.

Table 1. Oilseed, vegetable oil and crude oil market and production information

The Lipid Library	http://lipidlibrary.aocs.org/market/index.html
Oil World	http://www.oilworld.biz
USDA foreign Agricultural Service	http://www.fas.usda.gov
International Energy Agency	http://omrpublic.iea.org/
US Energy information Administration	http://www.eia.doe.gov/steo/contents.html#Global_Crude_Oil_And_Liquid_Fuels

Table 2. World production 2009/10 and typical fatty acid compositions of the nine major vegetable oils

Crop		Production MMT (2009/2010)	Oil composition (Fatty acid, % weight)						
Common name	Major cultivated species		< C16	16:0	18:0	18:1	18:2	18:3	> C18
Coconut	<i>Cocos nucifera</i>	3.7	80**	9	3	6	2		
Cotton seed	<i>Gossypium hirsutum</i>	4.7		22	2	21	54		
Olive	<i>Olea europaea</i>	3.0		12	3	73	9	1	1
Palm	<i>Elaeis guineensis</i>	45.0	2	44	4	39	10		1
Palm Kernel	<i>Elaeis guineensis</i>	5.5	71**	8	2	16	3		
Peanut	<i>Arachis hypogea</i>	4.6		10	3	48	30	1	7
Rapeseed *	<i>Brassica napus</i>	22.1		5	2	60	22	10	1
Soybean	<i>Glycine max</i>	37.9		10	4	18	55	13	
Sunflower	<i>Helianthus annuus</i>	11.1	1	6	5	22	66		
Total		138.6							

*Now predominantly low erucic, low glucosinolate cultivars. MMT, million metric tonnes.

** Predominantly lauric acid (12:0)

Table 3. Examples of major non-food uses for vegetable oil

Fuel (Biodiesel and Bio-Derived Synthetic Paraffinic Kerosenes (Bio-SPK, hydroprocessed vegetable oil))
Lubricants (Greases, liquid lubricants, fuel additives, mould release oils)
Hydraulic fluids
Surfactants
Printing Inks
Surface coatings (paints, varnishes)
Solvents (paint removers)
Adhesives
Polymers (fibres, resins, foams, films)
Plasticizers
Waxes and candles
Personal care products (Lipstick, perfume, hair-care products)
Pharmaceuticals
Dust suppressant

Table 4. Examples of oilseed crops grown primarily for non-food uses

Common name	Major cultivated species	Properties	Key component	Major use
Castor Bean	<i>Ricinus communis</i>	Hydroxy fatty acids	Ricinoleic acid*	Chemical feedstock
Linseed	<i>Linum usitatissimum</i>	PUFAs	Linolenic acid*	Drying Oil
HEAR cultivars	<i>Brassica napus</i>	VLCFAs	Erucic acid*	Chemical feedstock
Jatropha	<i>Jatropha curcas</i>	Common C18 fatty acids	--	Bio-fuel
Tung	<i>Alerites fordii</i> syn . <i>Vernicia fordii</i>	Conjugated fatty acids	α -eleostearic acid*	Drying oil
Camelina	<i>Camelina sativa</i>	Common C18 fatty acids, VLCFAs	--	Bio-fuel
Potential new crops now under development**				
Crambe	<i>Crambe abyssinica</i>	VLCFAs	Erucic acid*	Chemical feedstock
Rain Daisy	<i>Dimorphotheca pluvialis</i>	Hydroxy fatty acids	Dimorphecolic acid (9-OH 18:2 Δ 10,12 <i>trans</i>)	Chemical feedstock
Cuphea	<i>Cuphea spp</i>	Medium chain fatty acids	C8 to C14	Chemical feedstock
Lesquerella	<i>Lesquerella fendleri</i>	Hydroxy fatty acids	Lesquerolic acid (14-OH 20:1 Δ 11 <i>cis</i>)	Chemical feedstock
Coriander	<i>Coriandrum sativum</i>	Novel monounsaturated fatty acid	Petroselinic acid*	Chemical feedstock
Calendula	<i>Calendula officinalis</i>	Conjugated fatty acids	Calendic acid (18:3 Δ 8,10 <i>trans</i> , Δ 12 <i>cis</i>)	Drying oil
Euphorbia	<i>Euphorbia lagascae</i>	Epoxy fatty acids	Vernolic acid*	Chemical feedstock

* Structure shown in Figure 2. **Examples. PUFA, polyunsaturated fatty acid; VLCFA, very long chain fatty acid.

Table 5. Industrial applications of oils enriched in very long chain polyunsaturated fatty acids and their derivatives

Viscoelastic surfactants and high molecular weight anionic surfactants
Enhanced Oil Recovery Surfactants
Paving bed polymers
Erucamide-Slip-promoting, anti-blocking agent in manufacture of plastic films
Nylon 13,13 or Nylon 15,15
Polyurethanes, plastics and foams
Coatings and adhesives
Modified epoxide gels and resins
Composite materials
Cosmetic formulations
Silver behenate for film processing
High temperature lubricants (intact oil)